

Sulfated polysaccharides interact with fibroblast growth factors and protect from denaturation

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Keywords

differential scanning fluorimetry; fibroblast growth factor; protein stability; sulfated polysaccharide

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(Received 12 March 2019, revised 26 June 2019, accepted 3 July 2019)

doi:10.1002/2211-5463.12696

Fibroblast growth factors (FGFs) regulate embryonic development and homeostasis, including tissue and organ repair and specific aspects of metabolism. The basic FGF and acidic FGF, now known as FGF2 and FGF1, are widely used protein drugs for tissue repair. However, they are susceptible to denaturation at ambient temperatures and during long-time storage, which will reduce their biological activity. The interaction of FGFs with the sulfated domains of heparan sulfate and heparin is essential for their cellular signaling and stability. Therefore, we analyzed the interactions of FGF1 and FGF2 with four sulfated polysaccharides: heparin, dextran sulfate (DXS), λ -carrageenan, and chondroitin sulfate. The results of thermal stability and cell proliferation assays demonstrate that heparin, DXS, and λ -carrageenan bound to both FGFs and protected them from denaturation. Our results suggest heparin, DXS, and λ -carrageenan are potential formulation materials that bind and stabilize FGFs, and which may also potentiate their activity and control their delivery.

Fibroblast growth factors (FGF) regulate embryonic development and homeostasis, including the repair of tissues and organs and specific aspects of metabolism [1,2]. The basic FGF and acidic FGF, now known as FGF2 and FGF1, respectively, are the most studied FGFs and have been developed as therapeutics [1]. Along with the other paracrine FGFs, they bind to heparan sulfate (HS) chains and interact with FGF receptors (FGFR) (Fig. 1A). Activation of the FGFR by the binding of FGF ligand and HS co-receptor induces the activation of the FGFR kinase [3–5]. This phosphorylates its targets, leading to the activation of many intracellular signaling pathways, for example,

RAS-RAF-MAPK and PI3K-AKT, which regulate cell fate and specific cell activities [2,6–8].

Since FGF1 is the universal FGF ligand and so activates all FGFRs, and FGF2 activates ‘c’ isoform receptors, they possess multiple activities in development and in homeostasis, for example, mitogenic, neurotrophic and angiogenic activities [9,10]. Consequently, FGF1 and FGF2 have been developed as therapeutics for tissue repair. For example, FGF1 and FGF2 are used to accelerate wound healing in skin [1,11]. FGF1 and FGF2 (natural or *Escherichia coli*-derived) have potent activities on many cell types, but their activity, especially that of FGF1, reduces over

Abbreviations

CCK-8, Cell Counting Kit-8; CS, chondroitin sulfate; DMEM, Dulbecco’s modified Eagle’s medium; DSF, differential scanning fluorimetry; DXS, dextran sulfate; FGF, fibroblast growth factors; FGFR, FGF receptors; GalNAc, *N*-acetyl-D-galactosamine; HS, heparan sulfate; T_m, melting temperature.

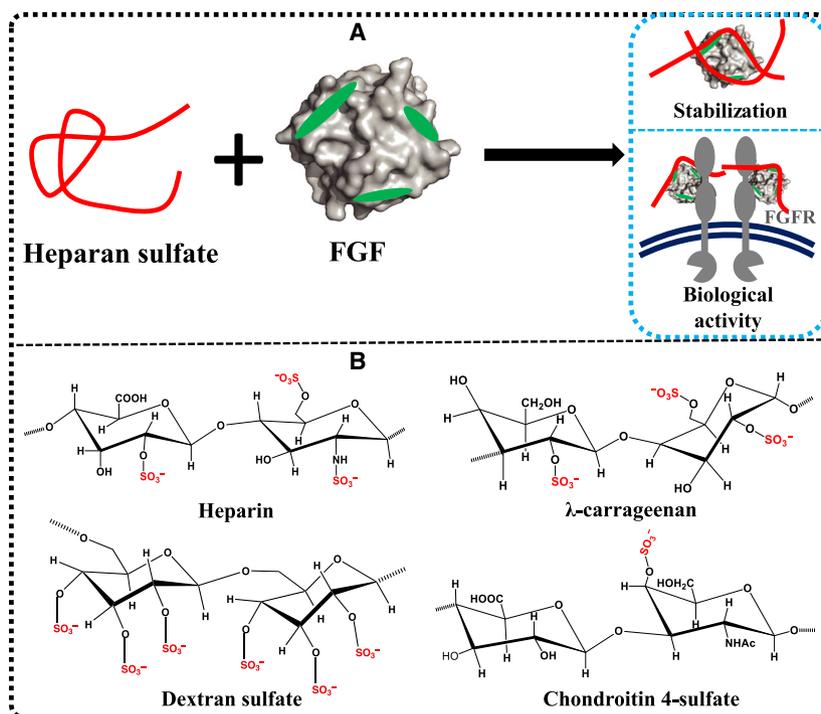


Fig. 1. Structural interaction of HS, FGF, and FGFR and the distribution of sulfate groups in heparin, λ -carrageenan, DXS, and CS. (A) FGF1 and FGF2 contain three HS binding sites in green which can interact with HS [19,32]. This interaction can stabilize the FGF protein and form a stable complex with FGFR competent to generate signals in the cytoplasm. (B) Structures of the disaccharide units of heparin, λ -carrageenan, DXS, and CS with sulfate groups in red. For heparin, this is the structure of the most common disaccharide unit, and 25% of disaccharides have fewer sulfate groups. HS has the same disaccharide structure as heparin, but is less sulfated [33,34].

time, even at body temperature [12–16]. Temperature-dependent denaturation and degradation by enzymes are likely to contribute to limiting the efficacy of these FGFs [17–21].

As well as enabling the formation of an active signaling complex with the FGFR (Fig. 1A), the interaction of the paracrine FGFs with heparin (Fig. 1B) or the related physiologically relevant polysaccharide, HS, increases their stability, including resistance to heat-induced denaturation and proteolysis [22]. Binding to HS also regulates their diffusion from source to target cell in tissues, and so their gradients and bioavailability [23–25]. A large body of work demonstrates that the interaction of FGFs with HS/heparin is mainly mediated by the sulfate groups on the polysaccharides [26]. However, heparin is expensive and it also has strong anti-coagulation activity, and so is not suitable as an activity enhancing component of a FGF therapeutic formulation [27,28]. Another common commercial animal-derived sulfated polysaccharide is chondroitin sulfate (CS), which has a *N*-acetyl- β -D-galactosamine (GalNAc) amino sugar, beta 1–3 linkages and is less sulfated (Fig. 1B), and is considered to only bind weakly to just a subset of FGFs [19,29]. Two highly sulfated polysaccharides are dextran sulfate (DXS) (six sulfate groups per disaccharide unit) and λ -carrageenan (three sulfate groups per disaccharide unit) (Fig. 1B), which like CS are cheap

and, due to their higher sulfation, may bind FGFs and so be potential additives for FGF protein formulations [30,31]. In the present work, we aim to determine the stabilization effects of DXS and λ -carrageenan on FGF1 and FGF2 and the ability of these polysaccharide chains to protect the biological activity of these FGFs.

Materials and methods

Materials

The polysaccharides were purchased from Sigma (St. Louis, MO, USA, heparin, 18 kDa average molecular mass; λ -carrageenan) and Solarbio (Beijing, China, DXS, 500 kDa average molecular mass and CS, 5–20 kDa). Since CS is often a mixture of species, this was analyzed by NMR [35] (Fig. 2). The data indicate that it is 60% chondroitin 4-sulfate, 40% chondroitin 6-sulfate, with other polysaccharides at too low a level to be reliably detected. *E. coli* strain (BL21) for protein expression was bought from Transgen Biotech (Beijing, China). Heparin affinity gel (Bio-Rad, Hercules, CA, USA), nickel affinity gel, and SP Sepharose Fast Flow (GE Healthcare Life Sciences, Uppsala, Sweden) were used for FGF protein purification. SYPRO Orange dye and optical 96-well plates were from Thermo Scientific (Eugene, OR, USA). The 293T cell line was from the stem cell bank of the Chinese Academy of Sciences (Shanghai, China). For 293T cell culture, the following materials were

used: Dulbecco's modified Eagle's medium (DMEM, containing 4500 mg·L⁻¹ glucose and 4 mM L-Glutamine; HyClone Laboratories, Logan, UT, USA; GE Healthcare Life Sciences), FBS (PAN Seratech, Aidenbach, Germany), PBS, trypsin, and penicillin–streptomycin (all Solarbio); cell culture dishes and 96-well plates were from Corning (Oneonta, NY, USA) and the Cell Counting Kit-8 (CCK-8) for detecting cell proliferation was bought from Dojindo (Shanghai, China).

FGF protein preparation

FGF1 and FGF2 proteins with a hexahistidine tag were expressed and purified as described [14,36]. Briefly, FGF1 and FGF2 were expressed in BL21 cells at 18 °C for 18 h. The bacteria containing FGF protein were harvested and stored at -80 °C. The bacterial pellets were resuspended in lysis buffer (50 mM Tris/Cl and 0.6 M NaCl, pH 7.4), and the cells were disrupted by sonication on ice. Cell debris

and insoluble proteins were pelleted by centrifugation at 4 °C, 30 000 *g* for 30 min. The supernatant containing FGF protein was purified by heparin affinity-gel chromatography. The eluate was further purified with nickel affinity chromatography and SP Sepharose Fast Flow chromatography to remove any remaining impurities, and the purified FGF protein was stored at -80 °C. Purification was confirmed by SDS/PAGE.

Protein thermal stability assay

Heparin powder (500 mg) was dissolved in 50 mL PBS to prepare a 10 mg·mL⁻¹ heparin solution for differential scanning fluorimetry (DSF) assays. DXS (10 mg·mL⁻¹), λ-carrageenan (10 mg·mL⁻¹), and CS (10 mg·mL⁻¹) were prepared in the same way. Each polysaccharide was diluted with PBS to prepare a range of concentrations.

Differential scanning fluorimetry was used to detect the thermal stability of FGFs, as described [26]. The purified

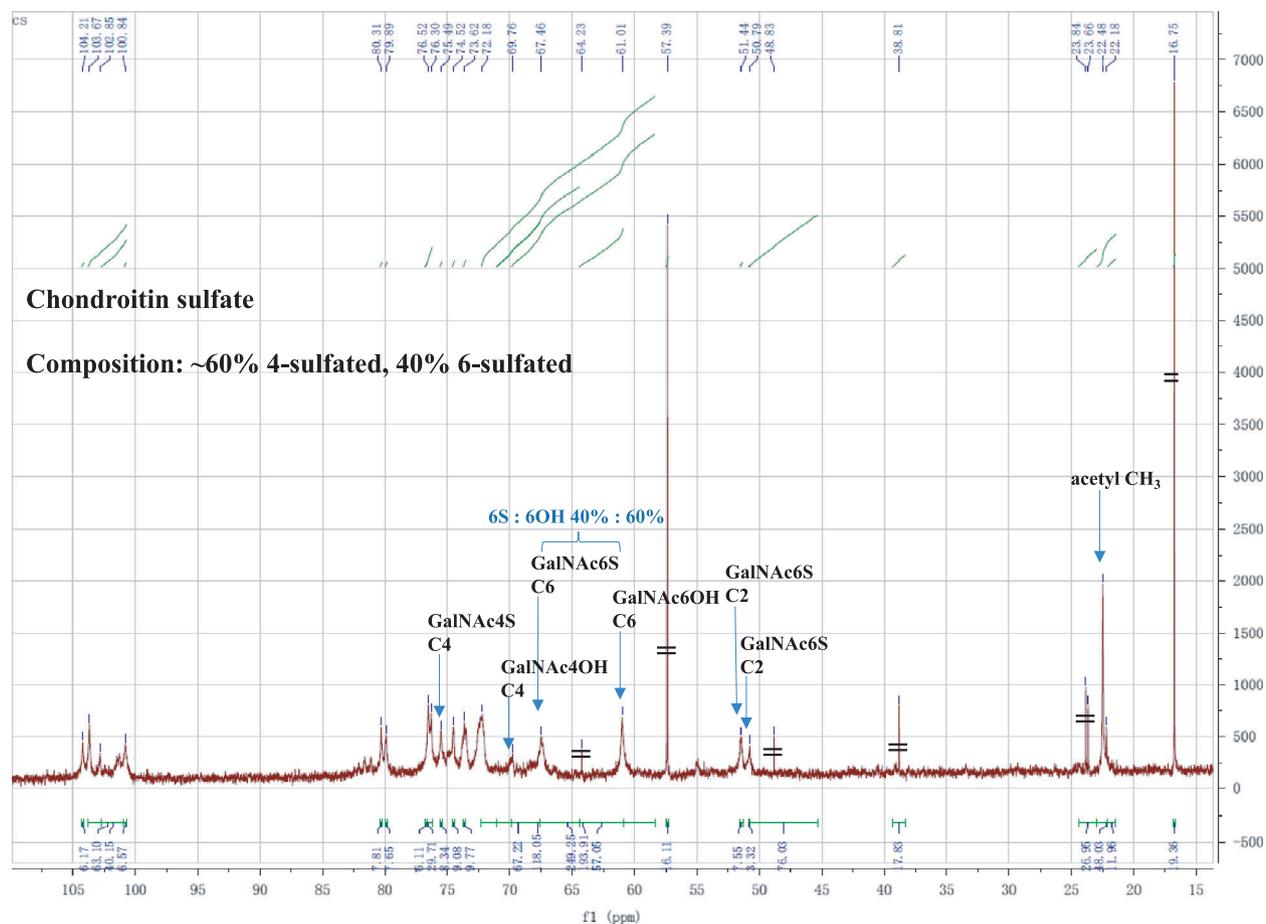


Fig. 2. Analysis of sulfation degree of CS. The C¹³ spectrum of the CS was analyzed with nuclear magnetic resonance (Acend 400 NMR, Bruker BioSpin, Bruker Scientific Technology Co., Ltd., Shanghai, China) by scanning for 20 h. The GalNAc signal comprises 40% 6-O-sulfate and 60% 6-OH groups, which indicates that the polysaccharide is a mixture of chondroitin 4-sulfate (60%) and chondroitin 6-sulfate (40%).

FGFs (5 μM), polysaccharide ligands (8 μL of a particular concentrations), SYPRO Orange dye (100 \times , 4 μL), and PBS (added to make up total volume to 40 μL ; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , and 1.8 mM KH_2PO_4 , pH 7.4) were mixed, and 10 μL added to each of three wells in an optical 96-well plate [26,37]. The plate was then covered with Optical Adhesive Film to prevent evaporation. The melting curve data were acquired with a QuantStudio™ 7 Flex Real-Time PCR machine (Applied Biosystems Life Technologies, Woodlands, Singapore). The running method was designed to raise the temperature from 32 °C to 81 °C (for FGF1) and to 92 °C (for FGF2) at a heating rate of 0.05 °C·s⁻¹. The raw data were exported for further analysis of melting temperature (T_m) after measurement.

DSF data analysis

The DSF data were analyzed with SimpleDSFviewer to obtain melting curves, first derivative curves and so the T_m [38]. The T_m was calculated by the half denaturation method [38]. The melting curve and first derivative curve were plotted in Excel, and the averaged T_m with standard deviation was plotted in MATLAB 2015a (The MathWorks, Natick, MA, USA).

Cell culture and cell growth assay

293T cells were cultured in DMEM containing 10% (v/v) FBS and 1% (v/v) penicillin–streptomycin. For cell growth assays, 293T cells were dispensed into 96-well plates at 2000 cells/well. After 24-h incubation, the cells were washed twice with PBS and cultured in DMEM containing 0.5% (v/v) FBS for another 24 h. FGF1, FGF2, and polysaccharides were added to the wells for 36 h, as described in the figure legends, after which CCK-8 (10 μL) was added to each for 2 h. The absorbance at 450 nm was then measured using a microplate reader (SpetraMax i3; Molecular Devices, San Jose, CA, USA).

To detect the thermal protection effect of polysaccharides, FGF1 and FGF2 were diluted to 400 ng·mL⁻¹ with DMEM with or without polysaccharide (1 $\mu\text{g}\cdot\text{mL}^{-1}$). The samples were incubated at 37 °C for 1 or 2 days and then added to cells in 96-well plates. The cell growth assay protocol was then followed; 293T cells were seeded into 96-well plates and starved with DMEM containing 0.5% (v/v) FBS (90 μL) for 24 h. Then, 30 μL of the pre-incubated samples of the FGFs was added to obtain a final concentration of 100 ng·mL⁻¹ FGF with or without polysaccharide in each well.

All of the acquired absorbance values (450 nm) for CCK-8 assay were normalized to the absorbance of the DMEM control group, and the results were analyzed and presented in MATLAB 2015a. Statistical analysis was performed with ORIGINPRO 2019 software (OriginLab

Corporation, Northampton, MA, USA). Multiple comparisons were analyzed by one-way analysis of variance with the Tukey test. $P < 0.05$ was considered significantly different.

Results and Discussion

Determination of interactions of FGFs with sulfated polysaccharide chains

FGF1 and FGF2 were purified by heparin affinity and nickel affinity chromatography. The melting curves of FGF1 and FGF2 (Figs 3 and 4) indicated a T_m of FGF1 of 49.5 °C and of 56.5 °C for FGF2. These data are entirely consistent with those previously published [15,26], which demonstrates that the purified FGF1 and FGF2 were correctly folded.

In the presence of heparin, the melting curve of FGF1 shifted to the right, indicating a stabilization of the protein through its interactions with the polysaccharide (Fig. 3A). This stabilization effect was first apparent at 20 $\mu\text{g}\cdot\text{mL}^{-1}$ heparin (T_m 51.8 °C, Fig. 3A) and reached a maximum at 2000 $\mu\text{g}\cdot\text{mL}^{-1}$ heparin (T_m 62.0 °C, Fig. 3A). Since λ -carrageenan and DXS are both highly sulfated polysaccharides, their ability to interact with FGF1 was tested. The DSF results indicate that FGF1 binds strongly to both λ -carrageenan and DXS, since it is stabilized by both polysaccharides (Fig. 3B,C,E). Moreover, the stabilization effect is concentration-dependent. The stabilization effect of λ -carrageenan was first observed at 100 $\mu\text{g}\cdot\text{mL}^{-1}$ (T_m 51.5 °C, Fig. 3B), and the T_m reached the maximum (T_m 60.5 °C, Fig. 3B) at 2000 $\mu\text{g}\cdot\text{mL}^{-1}$. Thus, the stabilization effect of λ -carrageenan is significantly weaker than that of heparin ($P < 0.05$). DXS possesses the highest sulfation level, and its stabilization effect was first apparent at 2.5 $\mu\text{g}\cdot\text{mL}^{-1}$ (T_m 50.8 °C, Fig. 3C). The melting curve then shifted to the right as the concentration of DXS was increased, and the T_m reached 64.3 °C at 2000 $\mu\text{g}\cdot\text{mL}^{-1}$ DXS (Fig. 3C). DXS was, therefore, more effective at stabilizing FGF1 than heparin. In contrast, CS did not have a detectable stabilization effect on FGF1, which may reflect its lower sulfation or different backbone (Fig. 3D) or both.

The DSF results indicate that FGF2 is a more stable protein than FGF1 (Figs 3 and 4), which is consistent with the previous studies [15,20]. The T_m of FGF2 increased at 5 $\mu\text{g}\cdot\text{mL}^{-1}$ heparin (T_m 58.1 °C, Fig. 4A) and was highest at 2000 $\mu\text{g}\cdot\text{mL}^{-1}$ heparin (T_m 80.3 °C, Fig. 4A,E). Similarly to heparin, λ -carrageenan also increased the T_m of FGF2 from 58.6 °C at 40 $\mu\text{g}\cdot\text{mL}^{-1}$ to 69.9 °C at 2000 $\mu\text{g}\cdot\text{mL}^{-1}$ (Fig. 4B,E). When FGF2 was stabilized by DXS, the melting curves showed two

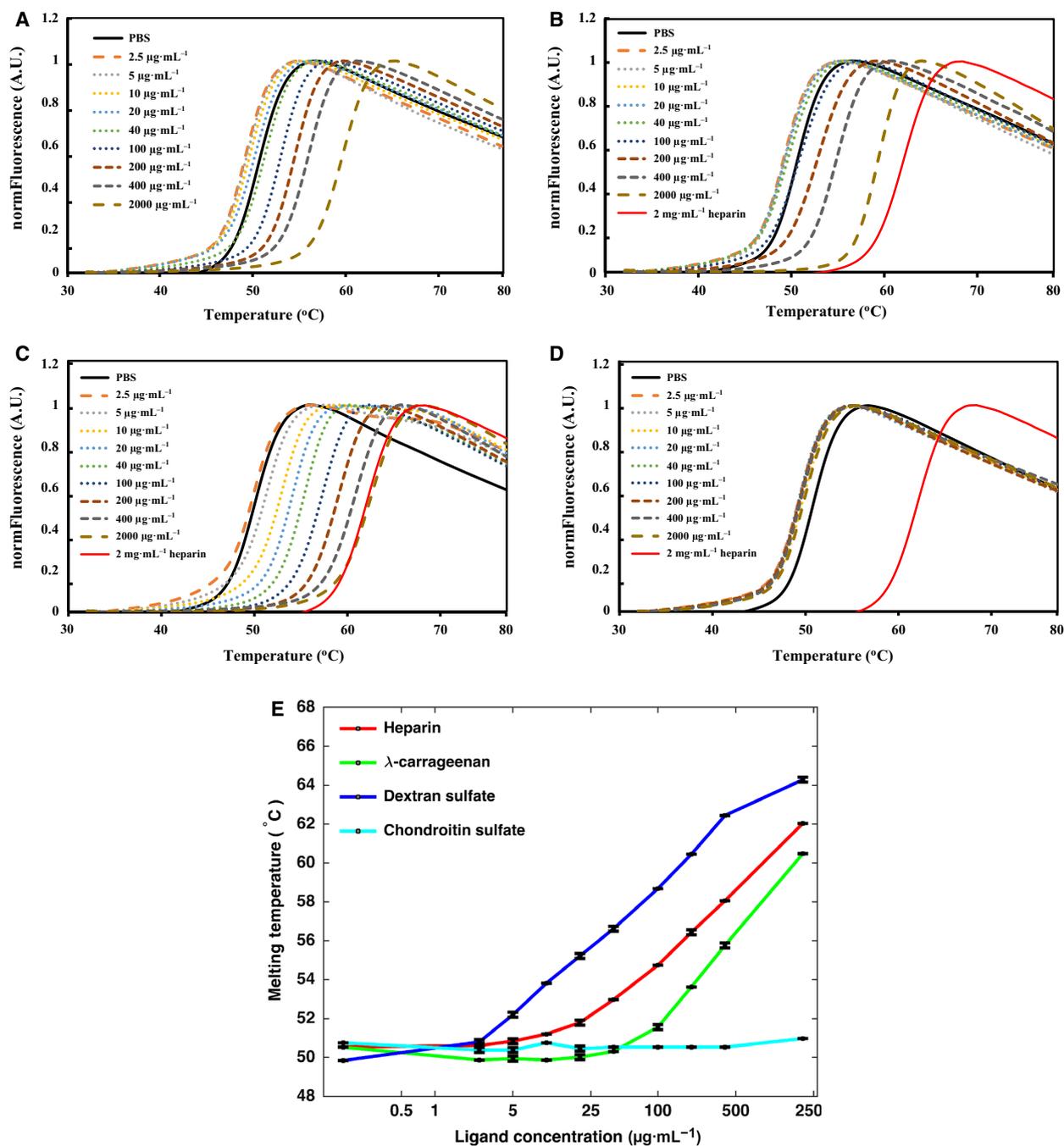


Fig. 3. Stabilization effect of polysaccharide ligands (heparin, λ -carrageenan, DXS, and CS) on FGF1. DSC of FGF1 (5 μ M) in the presence of varying concentrations of polysaccharide ligands (0–2000 μ g·mL⁻¹) in PBS (pH 7.4). Melting curve profiles of FGF1 with a range of heparin (A), λ -carrageenan (B), DXS (C), and CS (D). (E) Melting temperatures of FGF1 stabilized with polysaccharide ligands, mean of triplicates \pm SD.

FGF2 melting states (not stabilized FGF2 and polysaccharide-stabilized FGF2) (Fig. 4C,E). These indicate that over the timescale of the temperature change DXS does not dissociate from FGF2, so there is no averaging of the T_m. This effect is apparent, but much weaker

in the melting curves of FGF2 in the presence of heparin and λ -carrageenan. The stabilization of FGF2 by DXS was 77.2 °C (Fig. 4C). As with FGF1, CS did not have a detectable stabilization effect on FGF2 (Fig. 4D).

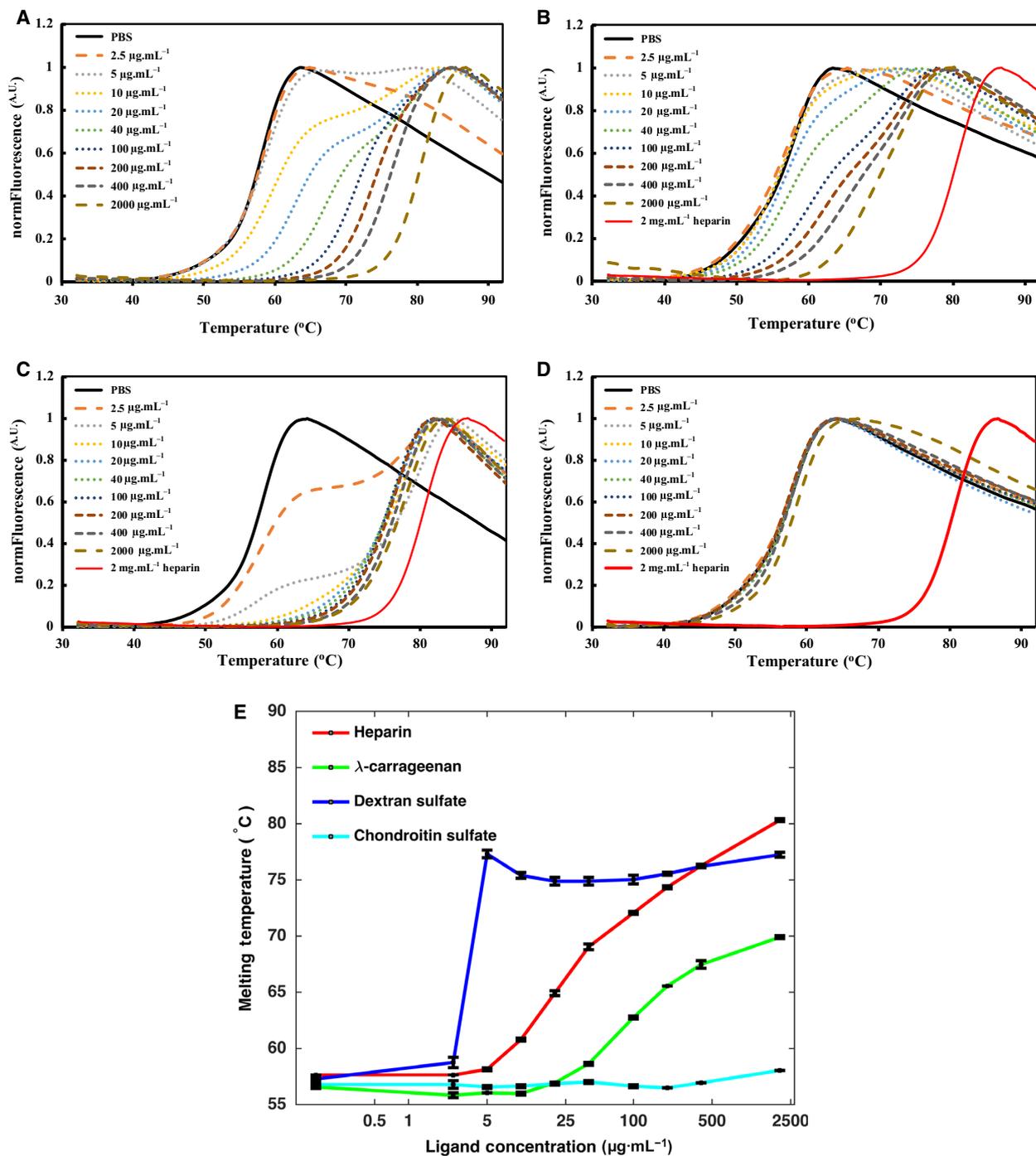


Fig. 4. Stabilization effect of polysaccharide ligands (heparin, λ -carrageenan, DXS, and CS) on FGF2. DSF of FGF2 ($5\ \mu\text{M}$) in the presence of varying concentrations of polysaccharide ligands (0 – $2000\ \mu\text{g}\cdot\text{mL}^{-1}$) in PBS (pH 7.4). Melting curve profiles of FGF2 with a range of heparin (A), λ -carrageenan (B), DXS (C), and CS (D). (E) Melting temperatures of FGF2 stabilized with polysaccharide ligands are the mean of triplicates \pm SD.

Stimulation of 293T cell growth by FGFs or polysaccharides

293T cells were used to measure the biological activities of FGF1 and FGF2 and of the polysaccharides.

When $0.03\ \text{ng}\cdot\text{mL}^{-1}$ FGF1 and FGF2 were added to culture medium, there was no change in cell growth (Fig. 5A,B). However, cell growth was observed to increase at $0.1\ \text{ng}\cdot\text{mL}^{-1}$ FGF1 or FGF2 (Fig. 5A,B).

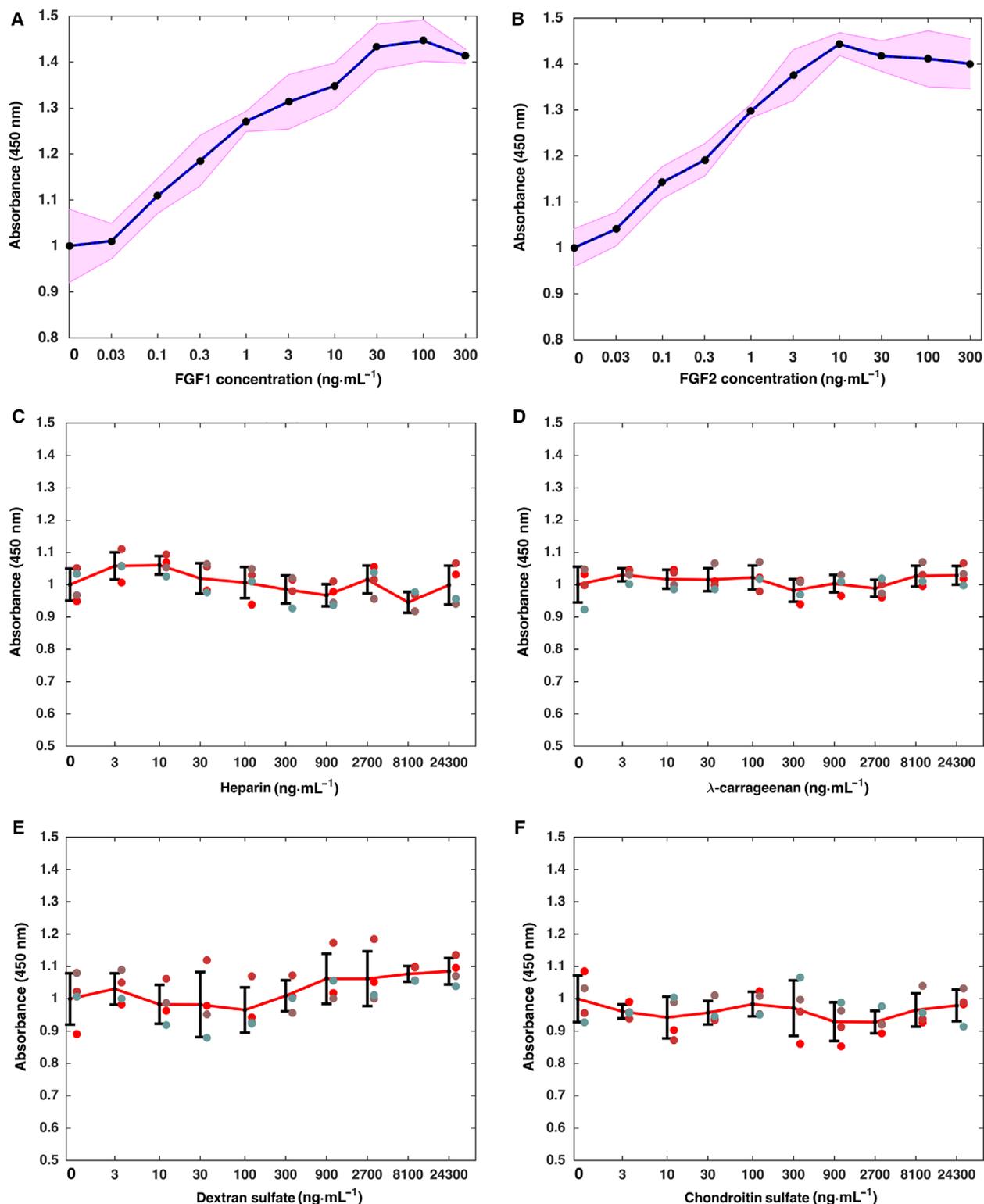


Fig. 5. Biological activity of FGFs and polysaccharides on 293T cells. 293T cells were treated with different concentration of FGFs (0–300 ng·mL⁻¹) and polysaccharides (0–30 000 ng·mL⁻¹) for 36 h, and the cell proliferation was measured with CCK-8. (A) FGF1; (B) FGF2; (C) heparin; (D) λ-carrageenan; (E) DXS; (F) CS. Results are the mean of quadruplicates after normalization, with the ±SD region shaded (A, B) or ±SD bars (C–F).

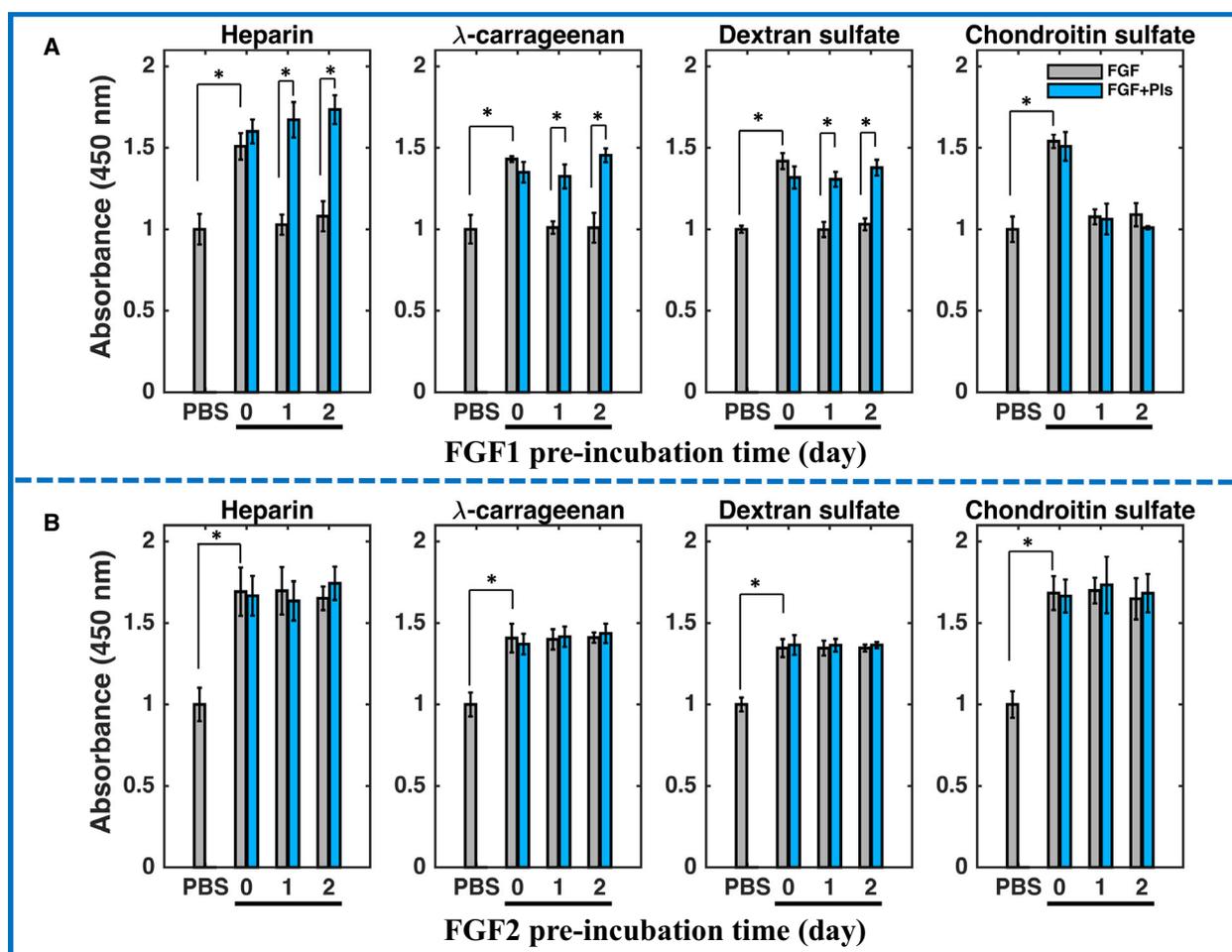


Fig. 6. Protection of biological activities of FGF1 and FGF2 by polysaccharides. FGF1 (A) and FGF2 (B) were pre-incubated with and without polysaccharide ligands (heparin, λ -carrageenan, DXS, and CS) for 0 day, 1 day, and 2 days at 37 °C. Their biological activities were measured in 293T cells by the CCK-8 assay, and data were normalized to the PBS control. FGF+PIs: FGF with the respective polysaccharide. Results are the mean of quadruplicates after normalization \pm SD and statistical analysis performed with the Tukey test. * $P < 0.05$.

As the concentration of the growth factors was further increased, so did cell growth, until a maximum was reached at 30 ng·mL⁻¹ FGF1 and 10 ng·mL⁻¹ FGF2 (Fig. 5A,B). These results are consistent with the NIH 3T3 cell growth assays of the same growth factors [39].

In the presence of heparin, λ -carrageenan, DXS, and CS alone, no effect on the growth of 293T cells was observed (Fig. 5C–F). Thus, these four polysaccharides by themselves have no growth-stimulatory or toxic effect on the cells.

Protection of FGFs' biological activities by polysaccharides

To detect whether the selected polysaccharides can protect the FGFs' stability and biological activity,

FGF1 and FGF2 (400 ng·mL⁻¹ in DMEM) were pre-incubated at 37 °C for 1 day and 2 days to mimic the effect of long-term exposure to body temperature. A final concentration of FGF1 and FGF2 of 100 ng·mL⁻¹ was chosen, since this elicited a maximal response after 2 days (Fig. 5A,B). The results demonstrate that FGF1 directly taken from the freezer (0 day pre-incubation) possessed significant biological activity on 293T cells comparing to the PBS control (Fig. 6A). After 1 day pre-incubation at 37 °C, the biological activity of FGF1 was no longer apparent, indicating the protein had likely denatured over this time.

However, when heparin, λ -carrageenan, and DXS were added, FGF1 was stabilized and even after the 2 days pre-incubation the FGF1 had the same biological activity as FGF1 taken directly taken from the

freezer (Fig. 6A). These results suggest that heparin, λ -carrageenan, and DXS, which bind FGF1 and increase its T_m (Fig. 3E) protect its biological activity. Addition of CS did not show a detectable effect on the protection of FGF1's biological activity, which is consistent with this polysaccharide not increasing the T_m of FGF1 (Fig. 3E). FGF2 is intrinsically a more stable protein than FGF1, as shown in Figs 3 and 4. Similarly to FGF1, FGF2 shows a strong stimulation of cell growth on 293T cells (Fig. 6B). Moreover, after 2 days pre-incubation at 37 °C the activity of FGF2 on 293T cells remained the same, which suggests addition of the four polysaccharides is not required for the protection of FGF2's biological activity.

Conclusion

FGF1 and FGF2 have very different thermal stabilities, as illustrated by their T_m measured by DSF, 49.5 °C for FGF1 and 56.5 °C for FGF2. Indeed, FGF1 has been considered to be in a molten globule state at 37 °C [40]. The thermal stability of FGF1 and FGF2 is substantially enhanced by binding to heparin (Figs 3E and 4E [26]). It is interesting that the stabilization of FGF1 by heparin has been observed in cultured cells, which have pericellular HS capable of binding and stabilizing the growth factor [41,42]. This is likely due to the lower affinity of FGF1 for HS, compared with FGF2 [41], such that a substantial amount of the protein will partition into the bulk culture medium rather than onto pericellular matrix HS. Heparin has a strong anticoagulant activity and is relatively expensive. However, we show here that other highly sulfated polysaccharides, DXS and λ -carrageenan, can bind to FGF1 and FGF2 and increase their thermal stability. Moreover, these are able to stabilize FGF1 and ensure that it retains its activity for longer times. Thus, biomaterials containing λ -carrageenan or DXS would be suitable for FGF drug delivery and would enhance the activity of FGF1, allowing lower doses to be used.

Acknowledgements

We acknowledge Nana Li and Jikui Wang who provided help at the start of this project in Xinxiang Medical University. Funding was provided by the Xinxiang Medical University (Project number: 300-505232).

Conflict of interest

The authors declare no conflict of interest.

Author contributions

CS, EAY, ZG, and DGF conceived and designed the project. CS, ML, PS, EAY, and MY performed the experiments and analyzed the data. CS prepared figures and wrote the paper. DGF reviewed and revised the drafts of the paper.

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